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(Article begins on next page)

1 **A broad-selective enzyme immunoassay for non-invasive stress assessment in African Penguins**  
2 **(*Spheniscus demersus*) held in captivity**

3

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26 **ABSTRACT**

27

28 We applied a direct competitive immunoassay for measuring corticosterone and glucocorticoid metabolites  
29 in feces (FGMs) as a non-invasive tool for monitoring the stress response of African Penguins (*Spheniscus*  
30 *demersus*) held in captivity in a zoological facility. The developed assay, validated in-house, proved to be  
31 rapid (the test could be completed in 90 minutes), sensitive (LOD for corticosterone  $0.2 \mu\text{g l}^{-1}$ , dynamic  
32 range  $0.75\text{-}75 \mu\text{g l}^{-1}$ ) and broad-selective, as it cross-reacted with the major corticosteroids, thus allowing  
33 the detection of excreted FGMs resulting from a biological stressor. Matrix interference, due to  
34 components of faecal samples, was overcome by diluting sample extracts (1+4 or 1+9, depending on  
35 sample features).

36 The assay enabled us to investigate the response to stress in five animals- three adult males and two adult  
37 females- over a period of 30 hours, and to identify the peak of FGM production as being 7-10 hours after  
38 the stressful event.

39

40 **KEYWORDS** Corticosterone, glucocorticoid, faecal metabolites, biological validation, class-selectivity

41

## 42 INTRODUCTION

43

44 Animals held in captivity are subject to a variety of physical, social, dietary, and ecological limitations that  
45 affect their welfare and behavior.<sup>1,2</sup> In recent years, several zoos and aquaria have intensified efforts to  
46 develop approaches and tools for assessing the well-being of captive animals, due to increasing public  
47 concern about animal welfare and ethical issues.<sup>3</sup> The monitoring of animal welfare has been regulated by  
48 national and international provisions aimed at assuring biological and conservation requirements of  
49 individual species (e.g. Italian Legislative Decree 73,<sup>4</sup> Council Directive 99/22/EC<sup>5</sup>). In addition, several zoo  
50 associations operate at national and international levels in promoting excellence in animal care and  
51 welfare, and in maintaining a high standard of animal husbandry, e.g. UIZA (Unione Italiana Giardini  
52 Zoologici e Acquari),<sup>6</sup> EAZA (European Association of Zoos and Aquaria),<sup>7</sup> and WAZA (World Association of  
53 Zoos and Aquariums)<sup>8</sup>.

54 A feasible way to monitor the welfare of animals held in captivity is represented by the evaluation of their  
55 response to stress caused by major constraints imposed by living in zoos. Exposure to stress usually results  
56 in an increased secretion of glucocorticoid hormones (GCs) from the adrenal cortex, and GCs are therefore  
57 commonly used as stress markers in human and vertebrate animals.<sup>9</sup> The predominant avian GC is  
58 corticosterone (B), a C-21 hormone produced by the adrenal glands, involved in diverse regulatory  
59 mechanisms, including: immune reactions, protein synthesis and degradation, and metabolic reactions. The  
60 corticosterone plasmatic level is considered a reliable marker of stress levels in birds.<sup>9</sup> Nevertheless,  
61 measuring corticosterone and related GCs in the plasma requires handling the animals for blood collection,  
62 which could elicit, in itself, a substantial rise in GC concentrations **in the blood due to the stress caused by**  
63 **the restraint and taking blood.**<sup>9</sup> *blood collection, maybe?*

64 An accepted alternative method for the evaluation of adrenocortical activity is the measurement of GC  
65 metabolites excreted in feces.<sup>10</sup> Although GCs are not actually present in faeces as they are metabolized in  
66 the liver, their metabolic products are excreted into the gut via the bile. Faecal glucocorticoid metabolite  
67 (FGM) levels have been demonstrated to reflect plasmatic GC concentrations, although as an integrated  
68 measure over the time, and after a variable time delay compared to the plasmatic GC response to the  
69 stressful event.<sup>10,11</sup> A major benefit of measuring FGMs is represented by the fact that faeces collection is a  
70 non-invasive practice, **thus which allows allowing extensive sampling campaigns, sample collection from**  
71 **small animals as well, and prevention of bias due to the sampling itself. Faeces can be easily collected,**  
72 **because there is no need to capture or handle the animals; therefore, repeated samplings from the same**  
73 **individual are possible** without affecting the animal's behavior.<sup>11</sup> However, as metabolic pathways involved  
74 in GC degradation are numerous,<sup>12</sup> and are influenced by several factors (including, but not limited to:

75 species, gender, age, reproductive status, season, etc.), predicting the nature and the chemical structure of  
76 targets is almost unachievable. For the same reason, developing specific antibodies for the excreted  
77 metabolites of each species, in order to set dedicated immunoassays, is often impractical.<sup>13,14</sup> Nevertheless,  
78 it has been argued that knowing the chemical identity of faecal GC metabolites in each species and for each  
79 condition is unnecessary.<sup>15</sup>

80 The most widely accepted method to assess the stress response in animals by FGM measurement is a  
81 practical approach, based on the development of immunoassays that exploit so-called broad-selective  
82 antibodies (namely polyclonal antisera able to bind a group of related substances rather than a defined  
83 compound), and the demonstration of the capability of these assays to reflect adrenocortical activity by a  
84 physiological and/or biological validation.<sup>11</sup> ~~An increase of the response of the assay~~ An increased response  
85 of the assay (expressed as an increase of FGM concentrations), following an appropriate stimulus, such as  
86 adrenocorticotrophic hormone (ACTH) challenge or a recognized biological stressor, is assumed to  
87 demonstrate the capability of the assay to reflect changes in the activity of the hypothalamic–pituitary–  
88 adrenal axes<sup>11,12</sup> and thus to ascertain stress. The physiological and/or biological validation legitimizes the  
89 application of the assay for a certain species, and for those individuals and stressful conditions for which it  
90 has been tested.<sup>11,16</sup>

91 The pre-requisite for developing a useful immunoassay is, therefore, the availability of antibodies able to  
92 bind the wider variety of GCs (*i.e.* having a broad selectivity). However, sensitivity is also crucial, mainly  
93 when FGMs have to be detected in minute quantities of faeces, such as those belonging to small avian  
94 species.<sup>14,17</sup> FGM assays have been validated for a multitude of species, including several birds.<sup>9,17-19</sup> Most of  
95 these studies tended to employ commercially-available radioimmunoassays or enzyme immunoassays  
96 primarily developed to measure cortisol or corticosterone, which usually only cross-reacted with a few  
97 other GCs (Table 1), not unexpectedly, as they were designed to selectively measure the target compound.

98 Specially developed immunoassays have also been described, based on antibodies aimed at measuring a  
99 specific faecal GC metabolite (*e.g.* tetrahydrocorticosterone<sup>20</sup>) or designed to be group-specific (*e.g.* 11,17-  
100 dioxoandrostanes<sup>11</sup>). Despite their selectivity profiles, all these immunoassays were shown to be able to  
101 measure an increase in FGMs that were artificially stimulated in physiological validation experiments. In  
102 addition, they have occasionally been applied for non-invasive investigation of the stress response induced  
103 by a specific constraint<sup>9,21,22</sup>. However, the conclusions drawn about the effect of a supposed stressful event  
104 on animals also depended on the responsiveness of the employed assay, or rather, the capability to detect  
105 the increased adrenocortical activity. A higher assay sensitivity (*i.e.* lower detection limits) would, of course,  
106 be desirable.

107 Therefore, the aim of this work was to establish a sensitive and rapid enzymatic immunoassay in the direct  
108 competitive format that exploits a broad-selective antibody towards GCs. Assay optimization was  
109 conducted to identify experimental conditions aimed at maximizing sensitivity, and the developed assay  
110 was subjected to in-house analytical validation. The assay was used to measure FGMs from African  
111 Penguins (*Spheniscus demersus*) held in captivity in a biopark (ZOOM Torino, Italy), to and test its suitability  
112 for non-invasive monitoring of stress levels in these animals.

113 The African Penguin is a marine bird endemic of to South Africa and Namibia. The current conservation  
114 status of this species is “Endangered”, and WHO/WHAT? is indicated in the Red List of Threatened Species  
115 of the IUCN (International Union for Conservation of Nature) because the wild population has dramatically  
116 decreased in recent years to less than 75-80,000 mature individuals.<sup>23</sup> Therefore, the African Penguin faces  
117 a great risk of extinction, and *ex-situ* conservation programs are becoming increasingly crucial. The African  
118 Penguin is a monogamous species with a complex behavioural repertoire,<sup>24</sup> and is exhibited in large groups  
119 in zoos and aquaria all over the world. To improve the health and general well-being of African Penguins  
120 held in captivity, the identification of stressful conditions is required, in order to develop mitigating  
121 strategies. To successfully achieve conservation of endangered species, it is important that captive facilities  
122 focus their efforts on welfare and health, which [riferito a chi?] involves minimizing and reducing stressful  
123 stimuli facing animals in captive environments.<sup>25</sup> Measuring glucocorticoids as an indicator of adrenal  
124 activity can help conservation biologists and animal managers understand the causes of poor welfare.<sup>26-29</sup>

125 Matrix interference due to the variability of the faeces collected from five adult African Penguins (three  
126 males, and two females) over 30 hours was studied and surmounted through appropriate sample dilution.  
127 Immunoreactive FGM concentrations, measured by the developed assay, were also compared to those  
128 obtained by means of a reference enzyme immunoassay, previously developed and validated for a different  
129 species of penguin, the Adélie Penguin (*Pygoscelis adeliae*).<sup>30</sup>

130

## 131 MATERIALS AND METHODS

132

### 133 Materials

134 Steroids (S, Table 1) were purchased from Steraloids (Newport, RI, USA), except for cortisol (F), which was  
135 obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), N,N'-  
136 diisopropylcarbodiimide (DIC), N-hydroxysuccinimide (NHS), and 3,3',5,5'-tetramethylbenzidine liquid  
137 substrate (TMB) were also purchased from Sigma-Aldrich. Horse-radish peroxidase (HRP) was from Roche  
138 Diagnostics (Milan, Italy). Dimethylformamide (DMF), methanol, Tris(hydroxymethyl)aminomethane (TRIS)

139 and all other chemicals and microtiter plates were obtained from VWR International (Milan, Italy). Rabbit  
140 polyclonal anti-3-(O-carboxymethyl)oxime-BSA antibodies were kindly supplied by G. Bolelli (Servizio di  
141 Fisiopatologia della Riproduzione, Policlinico S. Orsola, Bologna, Italy).

142 The hapten used for enzyme labelling was cortisol-3-(O-carboxymethyl)oxime (F-3-cmo, Figure 1) and was  
143 synthesized as previously reported.<sup>31</sup> F-3-cmo was then conjugated with HRP by the carbodiimide ester  
144 method.<sup>32</sup> The obtained F-HRP conjugate was stored at 4°C, with the addition of 33% (v/v) of glycerol. The  
145 diluted working solution was prepared daily in TRIS buffer (20 mM, pH 8.5, with 0.3M NaCl, 1% BSA, w/v,  
146 0.1% Tween 20, v/v).

147 Steroid stock solutions were prepared by dissolving the powders in absolute ethanol and stored at -20°C.  
148 Standard solutions were prepared by daily diluting the stock solutions with methanol:water (35:65, v/v).

149

#### 150 ***Competitive Enzyme-Immunoassay (EIA)***

151 The immunoreactive solid phase was obtained by coating wells with 150 µl of the antiserum directed  
152 towards cortisol diluted 1:10,000 (v/v) with carbonate/bicarbonate buffer (50 mM, pH 9.6), followed by  
153 overnight incubation at 4°C. Uncoated well surfaces were blocked with 300 µl PBS supplemented with 0.5%  
154 of BSA for 1 hour at room temperature. Wells were then washed using a 0.05% Tween 20 solution.

155 Calibration curves were constructed by adding 150 µl of F-HRP (1.5 mg L<sup>-1</sup>) to 50 µl of B diluted in aqueous  
156 methanol (35%, v/v) at concentrations ranging from 0 to 50 µg L<sup>-1</sup>. The mixture was incubated for 1 hour in  
157 immunoreactive wells, followed by washing, and colour development was then obtained by a 30 min  
158 incubation with TMB (200 µl per well). A volume of 50 µl of sulphuric acid (2M) was used as a stop solution,  
159 and absorbance was recorded at 450 nm. Unknown sample concentrations were measured by replacing the  
160 B standard solution with sample extracts diluted 1+1 with water as well as further dilutions of 1+4 or 1+9  
161 with aqueous methanol (35%, v/v). All standards and samples were measured in duplicate.

162 Unknown FGM concentrations were determined by interpolation on the calibration curve, where the signal  
163 was plotted against the log of analyte concentration. For each experiment, a calibration curve was  
164 determined by nonlinear regression analysis of the data from the standards, using the four-parameter  
165 logistic equation.

166 Relative cross-reactivity (CR) was evaluated by carrying out standard curves of the investigated compounds  
167 (S) in the same experimental conditions as B, except for the concentration interval, which was in the range  
168 of 0-5000 µg L<sup>-1</sup>, and was calculated as follows:

169 
$$CR\% = (IC_{50} B / IC_{50} S) * 100$$

170 where  $IC_{50}$  is the S concentration that causes 50% inhibition of the maximum observed signal.

171

## 172 ***Samples and sample preparation***

173 A total of 28 faecal samples from three adult males and two adult females were collected. The colony was  
174 observed from a distance (>5 m), to avoid disturbing the animals, by a researcher standing motionless  
175 outside the exhibit. After a defecation event, the researcher entered into the exhibit and gathered the  
176 expelled faeces. As urinal and faecal excretion are combined in birds, we only collected the faecal portion  
177 from droppings, which was distinguishable by color.<sup>33</sup> Faecal samples were collected into cryovials and  
178 stored immediately after collection at -20°C.

179 Fortified samples were prepared by adding 2.5, 10, and 40  $\mu\text{g l}^{-1}$  of B to three sample extracts, which had  
180 previously been tested as containing low levels of FGMs.

181 FGM extraction was carried out as reported in the literature<sup>32</sup> with the following modifications. Briefly,  
182 penguin faeces (which were contaminated with sand of the exhibit) were transferred to a 15 ml tube and  
183 extracted with 5 ml of methanol: water (70:30, v/v), by shaking on a rotary shaker for 30 min.

184 After centrifugation for 5 min at 3000 x g to remove sand and particulate matter, 3 ml of the clear  
185 supernatant was transferred to a weighted tube, and the amount of the extracted sample was obtained as  
186 the difference between the total weight of the extract and the weight of the extraction solvent.

187 Sample extracts were immediately stored at -20°C until required for analysis.

188

## 189 **RESULTS AND DISCUSSION**

190

### 191 ***Competitive Enzyme-Immunoassay analysis***

192 The polyclonal antiserum used to develop the assay was developed by stimulating an immune-response  
193 using an F conjugate. Nevertheless, cross-reactivity towards B was preliminary demonstrated to be 100%  
194 (Table 2); therefore this antiserum was deemed to be suitable for measuring GCs in general, and thus  
195 exploited to set the immunoassay.

196 Checkerboard assays using various combinations of antibody and enzyme tracer concentrations were  
197 carried out to select appropriate F-HRP and antibody dilutions for the direct competitive assay. A dilution of  
198 1:10,000 (v/v) of antiserum, and a concentration of 1.5  $\text{mg l}^{-1}$  of F-HRP were selected as being the most



199 suitable based on the lowest  $IC_{50}$  value . B standards were diluted in aqueous methanol, as FGM extraction  
200 from faecal samples typically involves a high percentage of this solvent, according to the literature.<sup>11</sup> The  
201 assay proved to be robust for methanol contents lower than 40%, whereas sensitivity decreased for higher  
202 solvent percentages. Dilution of B standards in TRIS buffer also negatively affected assay sensitivity and  
203 precision. Therefore, the ideal diluent for B standards was established to be methanol: water 35:65 (v/v).

204 Figure 2 shows a typical inhibition curve obtained under optimized conditions. The  $IC_{50}$  value of the assay  
205 was  $4.5 \mu\text{g l}^{-1}$ . The limit of detection (LOD) was calculated at 90% inhibition of the maximum signal ( $A_{\text{max}}$ ),  
206 and the dynamic range as the interval between 20 and 80% of  $A_{\text{max}}$ <sup>34</sup>, and were estimated to be  $0.2 \mu\text{g l}^{-1}$   
207 and  $0.75\text{-}75 \mu\text{g l}^{-1}$ , respectively.

208

### 209 **Selectivity of the assay**

210 According to the literature, tetrahydrocorticosterone (THB) is thought to be the main B metabolite,<sup>33</sup>  
211 however, this point is still debated, and several other possible metabolic products, characterized by very  
212 different chemical structures, have also been shown to be excreted in faecal samples of birds.<sup>12,15</sup> Möstl et  
213 al.<sup>12</sup> suggested at least seven possible metabolic pathways starting from B and resulting in the production  
214 of: 3-hydroxycorticoids, 11-oxocorticoids, 21-deoxycorticoids, 21-acid corticoids, 17-oxoandrostanes, and 6-  
215 hydroxycorticoids. However, faecal metabolites of GCs in birds have not been positively identified, and no  
216 data are available on this subject in the literature. Most authors used competitive immunoassays (RIAs,  
217 Radio Immuno Assays or EIAs, Enzyme Immuno Assays), developed for measuring corticosterone or  
218 tetrahydrocorticosterone, as tools to assess FGM levels<sup>9,17,30</sup>, on the basis of a demonstration that the assay  
219 is capable of detecting an increase in FGMs, when artificially induced by an appropriate biochemical or  
220 biological stimulus. An increased immune-response of the assay is interpreted as a consequence of the  
221 increased FGM concentration, regardless of the identification of the chemical compound responsible for  
222 the increase.<sup>15</sup>

223 Although responsiveness to induced stress of validated immunoassays for measuring FGMs is  
224 controversially related to cross-reactivity of the assay itself (*i.e.* the capability of detecting several different  
225 GCs), as is evident from Table 1, achieving broad-selectivity should be a major requirement for the  
226 analytical method to be applied for detecting FGMs. Therefore, the selectivity of a polyclonal antiserum,  
227 obtained by immunizing with a cortisol derivative modified in position 3 (Figure 1), was tested in response  
228 to a large number of steroid structures, which varied according to the substituents in position 3, 11, 6, and  
229 17, and according to the presence of insaturations at position 1-2 and 4-5, consistent with the hypothetical  
230 metabolic modifications which corticosterone can undergo. Relative cross-reactivities compared to B are  
231 shown in Table 2. Most steroids were recognized by the antiserum, at levels between 7 and 38%; among

232 these, surprisingly, testosterone demonstrated a high cross-reactivity (30%) despite substantial  
233 modification of the substituent at position 17 compared to B and F. Oxidation of the 11-hydroxyl to form  
234 11-oxosteroids determined a sharp decrease in binding, as manifested by the relatively low cross-reactivity  
235 of cortisone compared to cortisol, and prednisone compared to prednisolone; on the contrary, the  
236 substitution of the 11-hydroxyl with hydrogen did not seem to negatively influence the binding (as evident  
237 by comparing P and 11-hydroxyl-P). The addition of a substituent at position 6 slightly affected the  
238 recognition (CR of metil-prednisolone and prednisolone were 26% and 38%, respectively).

239 Otherwise, THB and THF showed absolutely no cross-reactivity. The lack of recognition of these compounds  
240 was mainly attributed to the hydroxyl substituent at position 3, whereas the saturated ring partially  
241 contributed to decrease cross-reactivity, as demonstrated by comparing CR% for the couple  
242 adrenosterone/androstanedione, differing in the insaturation of the A ring, which determined a limited CR%  
243 decrease (from 1% to 0.2%). Furthermore, androstenediol and androstan-3,17-diol, both having a hydroxyl  
244 at position 3, were not recognized, independently from the saturation state of the A ring. The applied  
245 extraction procedure could not exclude the presence of conjugates glucocorticoid metabolites, i.e.  
246 glucoronides at position 3. However, the conjugation of the hydroxyl substituent could contribute to  
247 reverse the decreasing of recognition.

248

#### 249 ***Penguin faeces analysis: analytical validation of the EIA***

250

251 Collection of faecal samples from African Penguins held in the exhibit of a park creates two main concerns,  
252 namely the limited amount of the sample available, and the presence of exogenous materials belonging to  
253 the exhibit, such as sand and pebbles. To address the first issue, the developed assay should be as sensitive  
254 as possible, and matrix interference should be counteracted without excessive sample dilution. To take into  
255 account the presence of spurious materials, quantitation of faeces was obtained by weighing a fixed  
256 volume of sample extracts, after centrifuging,, to remove undesired components, rather than weighing the  
257 samples themselves. To relate the quantity of measured FGMs to the sample amount, the contribution due  
258 to solvent weight was subtracted from the extract weight. The obtained sample weight was, in fact, the  
259 weight of the soluble or extractable portion of the sample.

260 The extraction protocol was taken from the literature<sup>30</sup> and applied without further optimization. Since  
261 samples were extracted with methanol/water 70/30, a 1+1 dilution with water was carried out to match  
262 the organic solvent content of samples with that of the B standards, and to preserve the sensitivity of the  
263 assay..

264 Furthermore, we observed that collected samples were very variable, not only in terms of the recovered  
265 amounts of faeces, but also in terms of the aspect of the extracts. Some extracts were intensely coloured  
266 (from pale yellow to dark green); some were transparent, while others were turbid, independently from  
267 regardless of the colour. The variable appearance of extracts was thought to be connected to faeces  
268 composition, and could depend on individual biological variability, individual circumstances at the time of  
269 collection, time elapsed from feeding, urea content, etc. Therefore, matrix interference on the assay was  
270 evaluated by carrying out recovery experiments on four representative samples: a turbid white (TW), a  
271 turbid orange (TO), a limpid light green (LG), and a limpid dark green sample (DG). Extracts were fortified at  
272 three levels with B (2, 10, and 50  $\mu\text{g l}^{-1}$ ) and non-fortified and fortified samples were analysed using the  
273 developed EIA. All samples were strongly overestimated, as testified by recovery rates that were two to  
274 ten-fold greater than the expected values (data not shown). The same samples were also tested after being  
275 diluted with water or with methanol/water 35/65 as follows: 1+0, 1+1, and 1+4. In addition, two buffered  
276 solutions (TRIS buffer at pH 8 and 9) were evaluated as the F-HRP diluent. The pH of the buffering solution  
277 and the methanol content did not significantly affect the results obtained on faecal samples (data not  
278 shown); nevertheless, dilution factors were demonstrated to strongly influence FGM estimation, mostly for  
279 samples that displayed green coloured extracts (Figure 3). The TW sample showed very low levels of FGMs,  
280 which resulted as undetectable at higher dilution factors, and were related to the scarce faecal material  
281 present in the sample, as confirmed by calculated weight (5 mg). Turbidity, likely associated to urea content,  
282 seemed to have a lesser effect on the reliability of results, compared to colour. Green coloured samples  
283 were more prone to matrix interference than yellow-orange samples.

284 To limit the matrix effect, and to establish a unique sample treatment, which possibly did not depend on  
285 the characteristics of the sample, an overall 1+9 dilution of faecal sample extracts was chosen, with the  
286 following diluents: 1+1 with water to reduce the organic solvent content and match conditions of the  
287 greatest sensitivity of the assay, followed by a further 1+4 dilution with methanol/water 35/65 (total  
288 sample extract dilution: 1:10). Nonetheless, very dark extracts were also analysed in a dilution of 1:20 (1+1  
289 with water and a further 1+9 with methanol/water), and FGM concentrations were calculated from the  
290 mean result of the two dilutions, when agreeing, otherwise from the value given by the higher dilution  
291 factor.

292 The accuracy of the optimized EIA method was investigated by means of recovery experiments on three  
293 faecal sample extracts, which were previously assessed to contain low levels of FGMs ( $< 500 \text{ ng/g}$ ), and  
294 were fortified at three B levels: 2.5 (low), 10 (medium), and 40  $\mu\text{g l}^{-1}$  (high). Within and between-assay  
295 precision was established by testing three faecal samples, which were shown to contain three  
296 concentration levels of FGMs (low, medium and high), in eight replicates from the same day, and on four  
297 different days, respectively (Table 4). Accuracy was between 83 and 116% (Table 3); within-assay precision

298 was measured to be in the range of 7-8% (n=8); and between-assay precision was measured to be in the  
299 range of 5-16% (n=4). The figure of merits of the optimized assay demonstrated that the developed EIA is  
300 accurate and precise enough to allow FGM determinations in penguin faecal samples, regardless of sample  
301 composition.

302

### 303 ***Biological validation of the EIA to assess adrenocortical response to stress***

304 To demonstrate the usefulness of the developed EIAs as a non-invasive tool for detecting adrenocortical  
305 response to stress in African penguins, a biological validation was carried out. Faecal samples from three  
306 adult males and two adult females were collected after a well-known cause of stress, namely the capture  
307 and immobilization of animals.<sup>35,36</sup> Sample collection started immediately after this stressful event, and  
308 continued until about 30 hours following the first collection, except at night. Sample frequency and  
309 numerosity depended on the individual, and ranged from three samples, from the animal named "G", to  
310 seven samples from the animal known as "S". The FGM content of each sample was measured by the  
311 developed v. The same samples were also analysed by the method validated by the group of Möstl and co-  
312 workers<sup>30</sup> for measuring FGMs in the faeces of Adélie Penguins (*Pygoscelis adeliae*) and Wilson's storm  
313 petrels (*Oceanites oceanicus*),<sup>20</sup> as a reference method. FGMs measured by both analytical methods are  
314 shown in Table 5, together with the time elapsed from the stressful event, and the amount of the sample  
315 available for analysis. As is evident, for some samples, a reasonable amount of faeces could be collected,  
316 whilst in other cases the available amount was lower or absent; FGMs were therefore only measured in the  
317 samples for which at least 20 mg of faeces were available (as recommended<sup>17</sup>).

318 Despite individual variability, results from all five animals qualitatively agreed in suggesting a peak of FGM  
319 production between 7 and 10 hours after the stressful circumstance. This observation is in good agreement  
320 with results previously reported for other birds. For example, Nagakawa *et al.* reported a profile of FGM  
321 excretion after ACTH administration which showed a peak after 6-18 hours in Adélie Penguins;<sup>30</sup> Denhard *et*  
322 *al.* observed a significant increase in FGM levels at 5.5-8 hours after ACTH administration to chickens  
323 (*Gallus domesticus*).<sup>13</sup>

324 In addition, the qualitative behaviour is in good agreement with results obtained through the reference EIA.  
325 Nevertheless, from a quantitative point of view, the developed EIA yielded higher levels of FGMs for all  
326 samples. The reference EIA, which used an antibody developed towards tetrahydrocortisone, generally  
327 gave lower FGM concentrations, and undetectable levels of FGMs in 12 out of the 22 samples analysed. The  
328 discrepancy between the two assays could be attributed to the different cross-reactivity profiles of the  
329 antibodies employed. The antibody used in the reference assay was decidedly more selective, as it only  
330 cross-reacted with 11-hydroxyandrosterone, tetrahydrocortisol, and cortol, while all other tested steroids

331 (Table 1) showed cross-reactivity values lower than 1%. Since FGMs are a group of unknown compounds  
332 that are structurally variable at different positions, as they could belong to several metabolic pathways, the  
333 broader the selectivity of the assay, the higher the probability of detecting a larger number of compounds  
334 and, therefore, the higher the sensitivity of the assay, or rather the capacity to identify the presence of  
335 FGMs.

336

## 337 **CONCLUSIONS**

338

339 We developed An enzyme immunoassay to detect glucocorticoid metabolites was developed, based on a  
340 broad-selective antibody. The assay was shown to be accurate, precise and decidedly more rapid than  
341 previously reported radio and enzyme immunoassays intended for measuring FGMs. Thus, the time needed  
342 to complete the analysis was 90 minutes, rather than overnight incubations, as required by existing  
343 immunoassays. The assay was applied to determine FGM levels from African penguins held in captivity, and  
344 demonstrated a reliable assessment of FGM increase solicited by an artificially induced biological stressor  
345 with high sensitivity. Indeed, ACTH infusion, which is the most commonly employed strategy to validate  
346 assays for FGMs, is a more efficient means to provoke the physiological increase of adrenocortical activity,  
347 and integrates the biological stress (capture, handling, injection) with the biochemical stimulus. However,  
348 as ACTH challenge is a potent stressor, the capacity of a proposed assay to detect the physiological  
349 response to stressful events could be overestimated by using this kind of inducement, thus limiting the  
350 reliability of conclusions drawn where less intense environmental, biological, or behavioural causes of  
351 stress are being investigated. The enzyme immunoassay developed in this study allowed the detection of  
352 an adrenocortical response to a biological stress (animal capture) in African penguins and demonstrated  
353 that the maximum physiological response (increase of FGMs) was reached after 7-10 h from the stressor.  
354 Therefore, this assay can be suggested as a reliable tool to evaluate the effect of potential stressful  
355 circumstances that these animals may undergo in captivity, such as, for example: visitor flow, excessive  
356 noise, and inappropriate weather. By identifying stressful stimuli, efforts can be made to reduce their effect  
357 and prevent their occurrence, in order to improve the general welfare of captive animals and increase  
358 breeding success. Nowadays, stress is one of the major issues facing zoological institutions around the  
359 world, and identifying and reducing sources of stress should therefore be a key factor for conservation  
360 programs of threatened species.<sup>25</sup>

361 We propose the use of the African Penguin as a model species, and the application of the same  
362 methodology to evaluate the well-being of other endangered species kept in captivity. Groups of African  
363 Penguins are housed in zoos and aquaria worldwide; these colonies are formed by a high number of birds,

364 enabling analysis of differences in age, gender and individuality. Finally, the ability to monitor  
365 adrenocortical activity in a non-invasive manner in African Penguins, and in general in endangered species  
366 held in captivity, is of major value in welfare management strategies, as prolonged periods of elevated GC  
367 concentrations interfere with numerous physiological processes, including immune and reproductive  
368 functions.

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